

Metabolism of One-Carbon Compounds by the Ruminal Acetogen *Syntrophococcus sucromutans*

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Syntrophococcus sucromutans is the predominant species capable of O demethylation of methoxylated lignin monoaromatic derivatives in the rumen. The enzymatic characterization of this acetogen indicated that it uses the acetyl coenzyme A (Wood) pathway. Cell extracts possess all the enzymes of the tetrahydrofolate pathway, as well as carbon monoxide dehydrogenase, at levels similar to those of other acetogens using this pathway. However, formate dehydrogenase could not be detected in cell extracts, whether formate or a methoxyaromatic was used as electron acceptor for growth of the cells on cellobiose. Labeled bicarbonate, formate, [1-¹⁴C] pyruvate, and chemically synthesized O-[methyl-¹⁴C]vanillate were used to further investigate the catabolism of one-carbon (C₁) compounds by using washed-cell preparations. The results were consistent with little or no contribution of formate dehydrogenase and pointed out some unique features. Conversion of formate to CO₂ was detected, but labeled formate predominantly labeled the methyl group of acetate. Labeled CO₂ readily exchanged with the carboxyl group of pyruvate but not with formate, and both labeled CO₂ and pyruvate predominantly labeled the carboxyl group of acetate. No CO₂ was formed from O demethylation of vanillate, and the acetate produced was position labeled in the methyl group. The fermentation pattern and specific activities of products indicated a complete synthesis of acetate from pyruvate and the methoxyl group of vanillate.

Acetogens have been the object of intense interest during the past few decades, especially with the observation that they often use a wide variety of substrates (23). Of considerable interest is their role in the anaerobic O demethylation of lignin monomers (6). *Syntrophococcus sucromutans* (21) was isolated as the predominant rumen species capable of O demethylation of methoxybenzenoids (10⁷ cells per ml of rumen fluid). Its unique physiology is characterized by an absolute requirement for an electron acceptor system to effectively metabolize a number of carbohydrates or pyruvate, with acetate being the final organic product and with part of the acetate synthesized from one-carbon (C₁) compounds. The reduction of the propenoate side chain of cinnamic acids, formate, or a hydrogenotrophic bacterium substitutes for the methoxyaromatic, thus serving as alternate electron acceptor systems.

To elucidate the pathways involved in the catabolism of C₁ compounds by *S. sucromutans*, the metabolism of various ¹⁴C-labeled C₁ substrates and enzymatic components has been studied in experiments using washed cells and cell extracts. Evidence is presented here that cells of *S. sucromutans* metabolizing pyruvate in the presence of formate or vanillate as an electron acceptor predominantly label the carboxyl group of acetate when labeled CO₂ or [1-¹⁴C] pyruvate is used. Conversely, the label from formate predominantly, as well as that from O-[methyl-¹⁴C]vanillate, almost completely labels the methyl group of acetate. On the basis of the results of the enzymatic investigation, the differences between *S. sucromutans* and other acetogens capable of metabolizing C₁ compounds by using the acetyl coenzyme A (acetyl-CoA) (Wood) pathway are further discussed (16, 23, 32).

MATERIALS AND METHODS

Source of organisms and maintenance. *S. sucromutans* S195 (DSM 3224) was from the laboratory collection of M. P. Bryant. Fructose and formate were replaced by cellobiose (10 mM) and syringic acid (5 mM), respectively, for maintenance, with a solid medium as described by Krumholz and Bryant (21).

Anaerobic techniques and medium preparation. Anaerobic techniques were used throughout (22). For growth of *S. sucromutans*, the medium of Krumholz and Bryant (21) was modified by the use of only 30 mM sodium bicarbonate under the same gas phase of N₂-CO₂ (4:1), and the replacement of Casitone (Difco Laboratories, Detroit, Mich.) by 0.2% (wt/vol) Trypticase (BBL Microbiology Systems, Cockeysville, Md.)-0.2% yeast extract. Phosphatidylcholine (type IX-E; Sigma Chemical Co., St. Louis, Mo.) was added as a lipid supplement in place of rumen fluid together with a mixture of volatile fatty acids (11). All incubations were at 39°C in the dark without shaking, and bacterial growth was measured spectrophotometrically (A₆₀₀; Spectronic 70, Bausch and Lomb).

Labeled vanillic acid preparation. Vanillic acid labeled in the O-methyl group was prepared by the method of Haider and Lim (18). Modifications to the procedure included the use of protocatechuic acid (PCA) ethyl ester as starting material and the use of ether extractions under alkaline and acid conditions in all steps following the recrystallization of PCA ethyl ester benzyl ether. The ¹⁴CH₃I (specific activity, 56 mCi/mmol; Amersham Corp., Arlington Heights, Ill.) was allowed to distill for 12 h in the reaction flask kept in an acetone-dry ice bath; then, 50 µCi was taken up. The final acid-ether extract was purified by preparative thin-layer chromatography using silica gel-coated glass plates (20 by 20 cm; Analtech, Inc., Newark, Del.). The upper phase of a solvent system containing toluene-ethyl acetate-formic acid (7:1:1) gave good separation of vanillate from the other

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products (one labeled constituent of the final products was believed to be isovanillic acid). After preparative thin-layer chromatography purification, the labeled vanillic acid was 96% pure by high-performance liquid chromatography. The concentration was measured spectrophotometrically (14), and the activity of the ethanolic solution finally prepared and used for assays gave a specific activity of 217,000 dpm/ μ mol.

Washed-cell experiments. The washed-cell suspensions for assays were prepared from a 3-liter batch culture in the desired medium (10 mM formate or 10 mM vanillin was used as a C_1 source) containing 10 mM cellobiose, 200 μ g of phosphatidylcholine per ml, and volatile fatty acids. Late-exponential-growth-phase cultures were harvested by using anaerobic conditions throughout. The cell pellet after centrifugation ($8,000 \times g$, 8 min, 20°C) was suspended in 200 ml of 100 mM potassium phosphate buffer (pH 6.8 and containing 5 mM cysteine) previously equilibrated under N_2 . The cell pellet was washed once and resuspended in 15 to 20 ml of the same buffer. This suspension was used immediately in assays (1 ml per 10-ml assay mixture). The assay mixtures were left for 3 h at 39°C in the dark and were gently swirled every 15 to 20 min. All of the assay components were prepared in anaerobic buffer under anaerobic gas, N_2 - CO_2 (4:1), and were preincubated for 30 min at the assay temperature before addition of the cell suspension via syringe. Two different types of assays were performed with time points at 0, 1, and 3 h. In the first type, 10-ml assay mixtures were incubated for 3 h in 25-ml Balch tubes with pyruvate, formate, and bicarbonate- CO_2 , 0.5 mmol each, in the same buffer used to harvest the cells. In both cases with either formate (2 μ Ci/mmol) or bicarbonate (0.5 μ Ci/mmol) as the labeled compound, one set of control assays was run. In the second type, 5-ml assays were incubated in 12-ml Balch vials with vanillate (0.025 mmol) and pyruvate and bicarbonate (0.05 mmol each). O-methyl-labeled vanillic acid (1.2×10^6 dpm) or carboxyl-labeled pyruvate (1.5×10^6 dpm) were used as labeled substrates. Assay conditions were otherwise identical. The difference in label in the complete acidified assay mixture and in the supernatant of a centrifugation ($12,000 \times g$, 15 min) was used as a measurement of the labeling of acid-precipitable material to assess total label recovery.

Assay mixtures were acidified with 1 ml of 3 N $HClO_4$ per 10-ml assay and shaken for 3 h at room temperature to improve the release of CO_2 . Controls for time zero were acidified prior to the addition of the cell suspension. CO_2 released was trapped in 2 ml of 3 N NaOH in an evacuated Balch tube connected with the reaction vial via a double-ended Vacutainer needle. Exchange was allowed to proceed for 3 to 4 h. A control with labeled bicarbonate was used in parallel to assess the recovery in this first exchange (90.6%). For counting, 0.2 ml of NaOH from the trap was acidified with 1 ml of 2 N HCl for exchange into 1 ml of phenylethylamine, and aliquots were counted directly. A setup was developed for this exchange with a test tube with rim and side arm (J. Doré, Ph.D. thesis, University of Illinois, Urbana, 1989). Up to 1 ml of phenylethylamine in the trap could be counted in 6 ml of Aquasol-2 with counting efficiencies of 95% or more. For analytical purposes, 1 ml of NaOH solution from the trap was treated by the method of Kornblatt et al. (20) for assay as $BaCO_3$.

For separation of acetate, formate, and pyruvate, the procedure of Thauer et al. (31) based on isoionic-exchange chromatography was used as described by Diekert et al. (10). For assays without vanillate, 1-ml resin beds (Dowex 2-X8) were poured and gravity elution was used, giving an average

flow rate of 9.25 ml/min. A 1.5-ml aliquot of the reaction mixture was neutralized by addition of 85 μ l of 3 N KOH and then incubated 1 h at 0°C and clarified by centrifugation ($10,000 \times g$, 10 min) before being loaded onto the column. Five milliliters of water was used to wash the column; acetate elution was started with 50 mM formic acid (15 1-ml fractions), followed by elution of formate and pyruvate with 1 M formic acid (30 1-ml fractions), and the remaining label was eluted with 4 M HCl (15 1-ml fractions).

For assay reaction mixtures with labeled vanillate, Sep Pak C_{18} cartridges (Waters Associates, Milford, Mass.) were first used. They were prepared by passage of two 5-ml amounts of methanol and then 50 ml of water through the cartridges. One-milliliter volumes of labeled vanillate and acetate standards or assay reaction mixtures with labeled vanillate plus 0.6 ml of 1 N KOH and, in the latter case, 0.1 ml of 0.5 M unlabeled sodium acetate were placed in the cartridge and then washed in with two 1-ml washes of water. One-milliliter fractions were collected starting with the second wash and throughout further elution with water at a flow rate of 2 to 3 ml/min. With the labeled standards, the first 2 ml contained no radioactivity and acetate eluted with the next 4 ml. Vanillate eluted in the next 30 ml, and the vanillate elution fractions were pooled for counting of radioactivity (98% recovery). With the labeled vanillate reaction mixtures, a small amount of vanillate was suspected to have coeluted with acetate. Therefore, the 4-ml amounts containing acetate were further purified by being loaded onto a 2.5-ml Dowex 2-X8 column, which was prepared as described above, and washed in with 10 ml of water. Acetate was then eluted (flow rate, 1.2 ml/min) as described above with fractions of 100 to 500 μ l. Radioactivity in acetate was then counted and prepared to determine the position of the label.

Acetate degradation for assay of the label in the carbon-1 (-COOH) and the carbon-2 (- CH_3) separately as CO_2 was done by the method of Abraham and Hassid (1), as modified from that of Phares (27). The overall recovery and recovery of individual carbons were assessed by using $1\text{-}^{14}C$, $2\text{-}^{14}C$, and uniformly labeled acetate controls, and assay data were corrected accordingly. Aliquots suspended by using Aquasol-2 were counted with a Beckman LS 5801 scintillation counter, and all counts were corrected for quench and background.

Pyruvate, formate, and acetate were assayed by high-performance liquid chromatography, as formerly described (8), with a Beckman model 334 high-performance liquid chromatograph equipped with an Aminex HPX87H column (300 by 7.8 mm; Bio-Rad Laboratories, Richmond, Calif.). The mobile phase was 5 mM H_2SO_4 , with a flow rate of 0.6 ml/min and detection at A_{210} . Vanillate and PCA were assayed in the same manner except that the mobile phase was 5 mM H_2SO_4 in 20% acetonitrile and A_{254} was used for detection (see Bio-Rad catalog). In this case, the other organic acids eluted in the void volume.

Enzymatic methods. For preparation of cell extracts, cells of *S. sucromutans* were grown under the same conditions described above for preparation of washed cells. The cells were harvested by centrifugation ($8,000 \times g$, 8 min, 4°C). The pellet was suspended in 100 mM potassium phosphate buffer at pH 6.80 (1 ml of buffer per 1 g [wet weight] of cells), and this suspension was passed through a French pressure cell at 52,400 kPa. The resulting suspension was centrifuged ($35,000 \times g$, 1 h, 4°C), and the supernatant was decanted for use as the cell extract. Transfers were performed in an anaerobic chamber, and the preparation was constantly

TABLE 1. Metabolism of labeled compounds by washed cells of *S. sucromutans* and label recoveries

Compound assayed ^a	Radioactivity (%) in:						Acetate recovered (%)	
	CO ₂	Formate	Pyruvate	Vanillate	Acetate	Other ^b	-CH ₃	-COOH
NaH ¹⁴ CO ₃ ^c	61.1	0.3	12.2	0	4.5	12.9	20	80
H ¹⁴ COONa ^c	11.4	28.0	2.4	0	2.5	23.1	74	26
[1- ¹⁴ C]pyruvate ^d	7.6	0	18.8	0	13.6	74.6	16	84
O-[¹⁴ CH ₃]vanillate ^d	0	0	0	13.3	38.6	47.6	97	3

^a Assays with labeled formate or bicarbonate contained pyruvate, formate, and bicarbonate (0.5 mmol each per 10-ml assay). The original label was 5.5×10^6 dpm of bicarbonate or 22×10^6 dpm of formate. Complete assays with labeled pyruvate or vanillate contained vanillate (0.025 mmol per 5-ml assay), pyruvate, and bicarbonate (0.05 mmol each). The original label was 1.2×10^6 dpm of vanillate or 1.5×10^6 dpm of pyruvate. Assays were incubated 3 h at 39°C in the dark and stopped by acidification with HClO₄. CO₂ was trapped in NaOH, and the fermentation products and substrates were separated as described in the text.

^b Relatively large amounts of ¹⁴C were recovered in the acid-precipitate fractions, and lesser amounts were recovered in the HCl strippings of the Dowex columns.

^c Cells grown on cellobiose-formate. Total recoveries of label were 91% (HCO₃⁻) and 67.6% (formate).

^d Cells grown on cellobiose-vanillin. Total recoveries of label were 114.6% (pyruvate) and 99.5% (vanillate) and 9.7% of recovery in other fractions was in PCA.

maintained under a stream of N₂ during the French press treatment. The supernatant after ultracentrifugation was decanted under N₂, distributed into Balch tubes in 1- to 2-ml amounts, and frozen at -20°C. Each tube was thawed only once just before use.

Formate dehydrogenase (FDH) was assayed as described by Ljungdahl and Andreessen (24), and strictly anaerobic conditions were maintained throughout. The assay components were mixed and dispensed in the anaerobic chamber, and preequilibrated plastic cuvettes or glass tubes were sealed with butyl-rubber stoppers. These mixtures were equilibrated at the assay temperature (37°C) with bubbling and flushing with N₂. To start the reaction, cell extract was added via syringe through the stopper with the plastic cuvettes and the glass tubes were inverted to mix the enzyme preparation added in a container, suspended midway above the liquid level, prior to flushing (P. Debeire, personal communication). Reduction of the electron acceptor was followed over time at A₆₀₀ for methyl viologen (MV) and A₃₄₀ for NAD and NADP. A pellet of *Eubacterium oxidoreducens* (22), provided by L. R. Krumholz, was used as the FDH-positive control.

Formyl-tetrahydrofolate (THF) synthetase was assayed as described by Buttlair (7), and methenyl-THF cyclohydrolase and methylene-THF dehydrogenase were assayed as described by Ljungdahl et al. (25). Methylene-THF reductase was assayed as described by O'Brien and Ljungdahl (26). For this assay, [5-methyl-¹⁴C]THF was prepared as previously described (13). Uptake hydrogenase was assayed as described by Drake (12). Assay components were dispensed in the anaerobic chamber, and the gas phase was exchanged thereafter by flushing through the stopper with 100% H₂ and with N₂ as a control. Assays were started by inversion of the glass tubes to mix in the enzyme preparation as described for FDH above. Carbon monoxide dehydrogenase (CODH) was assayed by a procedure identical to the hydrogenase assay except that the gas phase was changed to 100% CO with 100% N₂ as a control.

The formation of acetate from [5-methyl-¹⁴C]THF was assayed by the procedure of Ghambeer et al. (17) except that the assay was performed at 39°C instead of 57°C. A similar procedure was used to test for the formation of acetate from O-[methyl-¹⁴C]vanillate. In addition to pyruvate, reduced coenzyme A, ferrous ions, and dithiothreitol in phosphate buffer (50 mM, pH 7.0), the complete reaction contained 1 μmol (10 μl) of vanillate (217,000 dpm) from an ethanolic stock solution and 0.5 μmol of THF. Acetate and vanillate were separated by passage of the reaction mixtures through regenerated Sep Pak C₁₈ cartridges with water as an eluant.

RESULTS

Experiments with growing cells. In earlier studies of *S. sucromutans* (21), fructose, as the electron donor, was appreciably metabolized only in the presence of an electron acceptor system such as formate or vanillate. Early in the present study, these experiments and others, with cellobiose as the donor and formate or syringate as the electron acceptor, gave essentially the same results (data not shown).

Washed-cell experiments. In experiments without ¹⁴C label being considered, pyruvate was metabolized with little relationship to the electron acceptor system.

In assays with labeled bicarbonate (Table 1), total recovery was 91%. Minus the recovery in CO₂ and in other fractions (Table 1), the recovery in products was 17% and most of this was recovered in pyruvate and acetate, the former, presumably, via a CO₂-pyruvate exchange reaction. There was little conversion of CO₂ to formate. Of the label in acetate, 80% was in the carboxyl group.

In assays with labeled formate (Table 1), the overall recovery was 67.6% of the original label. Minus the formate not used (28%) and the label recovered in other fractions (23.1%), 16.5% of the original label was recovered in products. While recoveries in acetate and pyruvate were only 2.5 and 2.4%, respectively, of the original label added, the conversion or exchange of formate with CO₂ was much larger (11.4% of the original label) than in the experiment with labeled bicarbonate described above. Yet, in the labeled formate assay, 74% of the label in acetate was in the methyl group. In addition, the last fraction of isoionic chromatography eluted with HCl contained 6.2% of original label added as formate. It contained a compound exhibiting the same retention time as citrate by high-performance liquid chromatography under the conditions used for assay of nonaromatic acids.

With labeled vanillate, no labeling of CO₂ was detected (Table 1). In the complete assay, 38.6% of the original vanillate label was recovered in acetate. The total recovery of label was 99.5%, and singly labeled acetate tended to be formed, with 97% of the label recovered in the methyl group of acetate (Table 1). Furthermore, the specific activity of acetate after 3 h was about fivefold lower than that of the substrate vanillate, which is consistent with a complete synthesis of acetate from pyruvate and vanillate.

When carboxyl-labeled pyruvate was used in the assay (Table 1), total recovery of label was very high (114.6%), as was recovery in other fractions (74.6%), and the latter was essentially all in the acid-precipitable fraction. That recov-

TABLE 2. Synthesis of acetate from [5-methyl-¹⁴C]THF or O-[methyl-¹⁴CH₃]vanillate by crude extracts of *S. sucromutans*

Growth and assay ^a conditions	Initial methyl- THF (dpm) ^b	Conversion to acetate (%)	Initial vanillate (dpm) ^b	Conversion to acetate (%)
Cellobiose-formate, complete	161 × 10 ²	3.5	197 × 10 ³	1.65
Cellobiose-vanillin				
Complete	159 × 10 ²	27.3	197 × 10 ³	0.65
Minus pyruvate	190 × 10 ²	3.6	204 × 10 ³	0.17
Minus CoA	177 × 10 ²	9.4	197 × 10 ³	0.54
Minus Fe ²⁺	157 × 10 ²	21.0	199 × 10 ³	0.37
Minus CoA and THF			188 × 10 ³	1.13

^a The assays were incubated for 10 min at 37°C with the complete reaction containing pyruvate (30 μmol), dithiothreitol (10 μmol), ferrous ammonium sulfate (5 μmol), reduced CoA (3.3 μmol), THF (0.5 μmol) in assays with vanillate only, and potassium phosphate buffer (pH 7.0) (50 μmol) in a total volume of 1 ml. The crude extracts, 8.70 mg of protein for the formate-grown cells and 8.17 mg of protein for the vanillin-grown cells, were added to the reaction and preincubated for 10 and 20 min, respectively. The labeled methyl-THF (1 μmol) or vanillate (1 μmol) was added to start the reaction.

^b From assays without cell extract.

ered in products was only 7.6% in CO₂ and 13.6% in acetate. In the acetate, 84% of the label was in the carboxyl carbon.

Cell extract experiments. When cell extracts of *S. sucromutans* were tested for the synthesis of acetate from methyl-THF and pyruvate, good conversion of the methyl label of methyl-THF to acetate was observed (Table 2). Furthermore, the incorporation of the methyl label of methyl-THF into acetate with the extract of vanillin-grown cells was dependent on the presence of pyruvate and showed intermediate levels of conversion in the absence of reduced coA or ferrous ions.

When methyl-labeled vanillate was assayed, poor conversion of substrates was observed, even in the presence of pyruvate (Table 2). When pyruvate, reduced CoA, or ferrous ions were omitted, the efficiency of conversion was decreased with a high dependency on the presence of pyruvate as seen with assays with the methyl-labeled methyl-THF. With the cell extract from formate-grown cells and by using the complete assay, conversion of the methyl label of vanillate to acetate was more than twofold higher than that of the complete assay with the extract from vanillin-grown cells. Finally, the removal of reduced CoA did not affect the conversion as much as in the assays with methyl-THF, and when both THF and reduced CoA were removed, the highest conversion from the extract of vanillin-grown cells was obtained.

Activities in crude cell extracts were measured for the enzymes of the THF pathway, and some differences were observed from activities extracts of cells grown with formate or vanillin as the electron acceptor (Table 3). Formyl-THF synthetase showed a higher activity in extracts of cells grown with the aromatic substrate than in those grown with formate. Methylene-THF dehydrogenase was specific for NAD. Methylene-THF reductase had a high specific activity in the extracts from cells grown in the presence of formate. CODH activities were detected for both extracts. Uptake hydrogenase specific activities were much higher for the extract from cells grown with formate as electron acceptor as compared with those for the extract of vanillin-grown cells (39.27 versus 2.33 μmol · min⁻¹ · mg of protein⁻¹, respectively).

TABLE 3. Specific activities^a of enzymes of the THF pathway in cell extracts of *S. sucromutans* grown with cellobiose and different C₁ electron acceptor compounds

Enzymatic activity assayed	Sp act ± SD (n) with C ₁ electron acceptor for growth	
	Formate	Vanillin
Formate dehydrogenase	— ^b	— ^b
Carbon monoxide dehydro- genase ^c	2.91 ± 0.20 (3)	2.31 ± 0.13 (3)
Formyl-THF synthetase	2.30 ± 1.20 (4)	15.45 ± 0.03 (4)
Methenyl-THF cyclohydro- lase	0.47 ± 0.01 (3)	0.36 ± 0.02 (3)
Methylene-THF dehydro- genase ^d	8.13 ± 0.56 (3)	6.30 ± 0.17 (3)
Methylene-THF reductase ^e	0.29 ± 0.10 (3)	0.06 ± 0.01 (3)

^a Specific activity is defined as micromoles of substrate converted or product formed per minute per milligram of protein.

^b No detectable activity with MV as the electron acceptor and no activity difference with or without formate with NADP as the electron acceptor.

^c MV was used as the electron acceptor, and activity is given as micromoles of MV reduced per minute per milligram of protein.

^d NAD but not NADP was the electron acceptor.

^e Flavin adenine dinucleotide was used as the electron acceptor, and [¹⁴C-methyl]THF was used as the substrate.

DISCUSSION

The previous results of studies of growth and fermentation balance (21) indicated that the acetogen *S. sucromutans* has a unique energy metabolism; the present results, with ¹⁴C-labeling techniques and studies of certain enzymatic activities, show that it uses a modification of the Wood (acetyl-CoA) pathway (16, 23, 32, 33) to catabolize pyruvate, CO₂, formate, and the methoxyl group of vanillate. It is similar to most acetogens (29) because, like *Sporomusa* spp. (28) it has a gram-negative ultrastructure but is phylogenetically related to the gram-positive phylum of eubacteria (J. Doré and D. Stahl, manuscript in preparation). *S. sucromutans* was shown to contain all of the enzyme activities of the Wood pathway necessary for metabolism of C₁ compounds on THF carriers (Table 3). The specific activities of the enzymes were in the range expected from former studies of *Clostridium thermoaceticum* (5), *C. formicoaceticum* (26), *Acetobacterium woodii* (30), and *C. thermoautotrophicum* (9); this was especially so when these bacteria were grown under heterotrophic conditions. Formyl-THF synthetase had higher specific activity in crude extracts of cells grown with cellobiose and vanillin than with those grown with cellobiose and formate; it has been previously shown that extracts of cells of species that can use formate have higher specific activities for formyl-THF synthetase when formate is not present in the growth medium.

CODH activity of *S. sucromutans* was good both in extracts of cells grown with cellobiose-formate and in extracts grown with cellobiose-vanillin (Table 3). The presence of this CODH activity presented further strong evidence in favor of the contribution of the Wood pathway, and in addition, since this activity was present in extracts of cells grown with the methoxyl group of vanillin or vanillate, it suggested that all of the enzymes, except the methyltransferase system, were similar when O demethylation supplied the C₁ electron acceptor compound for the methyl group of acetate. No attempt was made to demonstrate the involvement of a corrinoid enzyme together with the CODH in the transmethylation system.

While CO does not serve as an energy source for growth of *S. sucromutans* in the presence of carbohydrate (21), it is

possible that it could serve as the electron donor in the absence of carbohydrate in growing cells or, more probably, in washed cells or cell extracts.

It was not possible to detect any FDH activity in *S. sucromutans* (Table 3), though the control FDH in *E. oxidoreducens* showed very good activity and FDH activity has been found in all other acetogens that use the Wood pathway. A lack of sufficient anaerobiosis was ruled out because of the positive results with *E. oxidoreducens* and because other oxygen-sensitive enzymes such as methylene-THF reductase, CODH, and uptake hydrogenase were detected. The medium used for growth of *S. sucromutans* contained all of the trace minerals and metals required for synthesis of various FDHs (2-4, 17, 34). The lack of rapid exchange between [^{14}C]formate and CO_2 (see below) supported the observation that FDH activity could not be detected.

With washed cells of *S. sucromutans*, a clearly unique catabolism of *O*-[methyl- ^{14}C]vanillate was seen (Table 1). There was no exchange of label between this methyl group and CO_2 , formate, or pyruvate though exogenous pyruvate, or, presumably, sugar was necessary for the catabolism of vanillate. Almost all of the label metabolized was present in the methyl group of acetate. Since this organism requires an organic electron acceptor system such as vanillate in order to metabolize carbohydrates or pyruvate, it clearly needs a catabolic pathway(s) to reduce its pool of reducing equivalents. It is unable to effectively use H_2 as an electron-sink product of carbohydrate or pyruvate unless grown in coculture with a hydrogenotroph. A pathway that would convert the methyl group of vanillate to CO_2 plus six protons and six electrons would further overload the reducing equivalents it must dispose of. This is a major difference between *S. sucromutans* and acetogens, such as *C. thermoaceticum* (34) TH001 (15), *A. woodii* (6), and others (23), that can use the *O*-methyl group(s) of vanillate and similar compounds as sole energy sources to produce acetate and CO_2 .

At least one reason for the failure of *S. sucromutans* to produce CO_2 from the methyl group of vanillate might be its lack of FDH activity. The *O*-demethylating enzyme system (33) might transfer the methyl group to CH_3 -THF and hence, via transmethylation (23), to CODH and then to the methyl group of acetyl-CoA. The lack of FDH may stop any formate that might be formed by reversal of the THF pathway from CH_3 -THF from being used in CO_2 production and also would stop any CO_2 produced via reversal of CODH from metabolism via formate.

A concurrent reason for the failure of *S. sucromutans* to produce CO_2 from the methyl group of vanillate might be that the methyl transfer system could be very closely linked to the acetate-forming system with the involvement of metabolism and transport through the cell membrane systems. The intact washed cells showed good conversion of the methyl group of vanillate to the methyl group of acetate (Table 1), but the crude extracts (Table 2) of either cellobiose-formate- or cellobiose-vanillin-grown cells showed poor conversion of the methyl group to acetate. However, the methyl group of methyl-THF was much more readily converted to acetate by the same crude cell extracts. Thus, the intact cell membrane(s) seemed to have greater involvement in catabolism of vanillate than with CH_3 -THF, a presumed intracellular intermediate. Sensitivity of the *O*-demethylating enzyme system of *C. thermoaceticum* to cell fractionation has been noted by Drake (33) (personal communication).

The catabolism of the electron acceptor ^{14}C -formate by

washed cells was less clear than that of the methyl group of vanillate. Much label remained in formate, some label was found in CO_2 and pyruvate, and in the acetate formed, only 74% was found in the methyl group. The results indicated that there was not rapid interconversion of carbon between formate and CO_2 , as would occur in an organism having an active FDH (4, 16, 28). Some bacteria, such as *A. woodii* (30), have an active FDH and can totally synthesize acetate from either formate or H_2 - CO_2 .

The metabolism of $\text{NaH}^{14}\text{CO}_3$ by washed cells of *S. sucromutans* was another indication that the C_1 metabolism was different from other acetogens possessing the Wood pathway. Only a small amount of label was recovered in formate, again suggesting that there was little or no activity of FDH to allow rapid formate- CO_2 exchange. Some of the label was recovered in pyruvate, and in the acetate formed, 80% was in the carboxyl carbon.

In crude extracts from cellobiose-vanillin-grown cells, [methyl- ^{14}C]THF conversion (Table 2) indicated the pyruvate requirement and suggested a coenzyme A requirement and, to a lesser extent, an Fe^{2+} requirement for acetate production. This seemed to be similar to results with *A. woodii* (30). Conversion of *O*-[methyl- ^{14}C]vanillate to acetate by crude extracts of either cellobiose-formate-grown cells or cellobiose-vanillin-grown cells was much less than with labeled methyl-THF (Table 2), as indicated above. The higher incorporation of label from vanillate in crude extracts of cellobiose-vanillin-grown cells into acetate in assays minus CoA and THF than in those minus only CoA suggested that added THF might be somewhat inhibitory to conversion of the methyl group of vanillate to acetate.

In conclusion, although a better understanding of the metabolism of *S. sucromutans* has been provided herein, many questions remain concerning its sometimes unique metabolic features. For example, much more research is needed on the hydrogenase(s) and its natural electron carrier(s), the pathway of electron flow from the putative glyceraldehyde-phosphate dehydrogenase, the pyruvate dehydrogenase and its electron acceptor, the *O*-demethylating enzyme system and the sequence of methyl carriers, the transmethylation, and the possibility of cytochrome involvement or Na^+ requirement in certain enzyme systems (ruminal anaerobes almost always have moderate Na^+ requirements for growth). These types of studies should assist greatly in allowing us to understand the unusual formate and methoxyl "respiration" and syntrophic growth with a formate- H_2 -using methanogen during growth with carbohydrate as the electron donor.

The unique characteristics of *S. sucromutans* may have been dictated by the features of its ruminal environment. Intercellular H_2 concentrations in the rumen are about 1 μM except for shortly after the animal is fed, and the apparent K_m for H_2 is about 1 μM (19). For formate, the average concentration is 12 μM and the average apparent K_m is 30 μM (19). We need to obtain similar ruminal values for methoxybenzenoids. We also need to determine apparent K_m s for formate and compounds such as ferulate, vanillate, and syringate in *S. sucromutans*. These types of information should give us a much better idea of the major electron acceptors used by *S. sucromutans* in the rumen.

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